

7/12/56

Bay

Preparation of Benzyl-Methionine  $\pm$  DCC

750 mg L-methionine and 350 mg ASP were dissolved with 1 ml water and then added 4 ml pyridine followed by addition of 0.9 ml 8N HCl. Almost all of the ASP dissolved but there was still methionine crystals visible to this solution (in a microwave bath) added 5 gm DCC dissolved in 5 ml pyridine removed 0.5 g sample and added to 0.5 ml 1N NH<sub>4</sub>OH then added 1 ml FeCl<sub>3</sub> of filter and read at 540 m $\mu$ .

<u>Time</u>	<u>D<sub>540</sub></u>	<u>nm/ml</u>	using 0.340 $\mu$ M methionine hydrochloric acid At 60 mm added 2.5 gm DCC as a solid.
15 mm	305	18.0	
45	630	37.1	
60	910	53.5	
130	910	53.5	
150	950	55.9	

At 160 minutes volume of rx mixture was 18 ml  
If above correct then 1.00  $\mu$ M or completion.

At this point added 25 ml of H<sub>2</sub>O (rm temp) stirred for 10 min then filtered by suction (took about 20 minutes). Volume was 40 ml.  
Assayed for Hx  
0.05 ml  $D_{540} = .135 = 8 \mu\text{M/ml} \times 40 = 320 \mu\text{M}$

Seems as if most of the material was degraded during the filtration etc.

Bergy

7/19/56

Reaction of L-methionine & DCC

750mg of L-methionine dissolved in 1.4ml H<sub>2</sub>O. Added 4ml pyridine then 0.9ml 8N HCl. Stirred in ice bath then added 151 gms DCC dissolved in 5ml pyridine. took samples as before

<u>Time</u>	<u>D<sub>540</sub></u>
30'	018
60'	070
120	.057

Does not seem to be any significant amount of hydrolysis reaching material in the absence of the acceptor (ASP)

7/14/56

Formation of Acetyl-L-methionine using less methionine  
 Meth / ASP = 25/1

110mg ASP <sup>(0.3mM)</sup> + 110mg L-methionine (0.75mM) mixed with 0.5ml H<sub>2</sub>O  
~~110mg ASP + 110mg L-methionine~~ Add 0.7ml pyridine Then 0.12 ml 8N HCl Stir in  
 ice bath then add 2gms of DCC in 2 ml pyridine.  
 Analyzed 0.05ml by hydrolysis as usual.

<u>Time</u>	<u>D<sub>500</sub></u>	<u>μM/ml</u>
30	0.367	21.6
60	0.461	27.2
180	0.782	46.0

Total volume of rx mixture  
 6.7ml x 46.0 = 308 μM

Started = 317 μM ASP

At end of 5 hrs + 40 minutes took sample  
 at read 585 = 230 μM  
 Same breakdown.

7/16/56

### Synthesis of Acetyl-L-methionine using Slight Excess of L-methionine (.31 mM)

110 mg Asp + 60 mg L-methionine (0.4 mM) mixed with <sup>0.55</sup> ml H<sub>2</sub>O. Added 1.7 ml pyridine and then 0.06 ml 8N HCl. Stir in ice bath. Add 2 gm DCC in 2 ml pyridine.  
Analyzed 0.05 ml aliquots @ 112001 + 7613

Time	D <sub>540</sub>	$\mu\text{M}/\text{ml}$	Total (vol 6.5 ml)
30'	.338	19.7	128
60'	.442	26.0	169
120'	.741	43.6	284
210'	.718	42.3	275

They 310  $\frac{284}{310} = 92\%$

### Synthesis of Acetyl-L-methionine @ Methionine Limiting

110 mg Asp (.31 mM) + 22 mg L-methionine (0.14 mM) mixed @ 0.55 ml H<sub>2</sub>O. Added 1.7 ml pyridine. Then added 0.06 ml 8N HCl. Stir in ice bath and add 2 gm DCC in 2 ml pyridine.  
Analyzed as above

Time	D <sub>540</sub>	$\mu\text{M}/\text{ml}$	Total.
45'	.137	8.1	53
105'	.393	23.1	150
150	.373	22.0	143

7/16/62

Summary:

<u>Mols Meth/ mols Asp M1</u>	<u>Ratio</u>	<u>Water/ pyridine</u>	<u>Dec/ Meth</u>	<u>Yield</u>	
5/1	5	2.3/9	25/5 = 5	100%	(based on AMP)
5/0	-	2.3/9	25/5 = 5	0	"
0.15 0.31	2.5	0.62/3.7	10/15 = 13	97%	"
0.4/0.31	1.3	0.61/3.7	10/4 = 25	92%	"
0.15/0.31	0.48	0.61/3.7	10/15 = 67	100%	(based on Meth)

7/13

Prep of Acetyl-Meth

350mg ASP + 160mg L-methionine mixed in 1.1ml H<sub>2</sub>O. Treated  
 in exactly same way as described on 7/19. However at end  
 realized added 60mg methionine instead of 160.  
 Total base volume 13.2

1.1ml	2540		
60'	245		
120'	475		
180'	499	29.4	382 $\mu$ M

Actually put in 400  $\mu$ M

7/19/56

Synthesis of Adenyl in methanone

1.1ml H<sub>2</sub>O + 3.5ml Pyridine  
 350mg ASP (free acid) 10mM + 160mg L-methionine (1.07mM) mixed with  
 Added 0.15ml 5N HCl. Cool with ice bath & magnetic stirrer. Add 4g DCC dissolved in 4ml pyridine (vol ca 25).  
 Entire mixture stirred in ice-bath. Removed 0.05ml samples add to 0.95ml 1M NaOH (pH > 7). Then add 1ml Fracis opt. slide, filter & read vs blank at 540mμ

Time	D <sub>540</sub>	μM/ml
85'	.481	28.3
180'	.982	57.8
240'	1.050	61.8
270'	1.166	68.7

Vol 13

At 300' added 100ml of acetone at ca -20° and let sit in ice bath for 5 min. Heavy coarse ppt settled out. This was filtered by suction and the ppt washed 2x in 100ml portions of 75% acetone. The washed & ca 150ml of the solid washed & placed in vacuum desiccator for ca 1 hour. Yield dry powder 650mg.

weighed out 49 mg and dissolved in 2ml <sup>cold</sup> 0.0005N HCl

filtered  
 Assay by Hx.

Wavelength	D <sub>540</sub>	μM/ml
0.10	379	11.1
0.20	758	11.1

} 11.1 x 2.1 = 23.3 μM/49mg

Spectrum on dilute acid solution

Diluted 0.03 → 10.0ml in 0.01M HCl.

λ	D	Ratio
250	1.207	240/260 = 0.2
255	1.433	250/260 = .86
260	1.402	Based on ratio of 0.22 E <sub>260</sub> = 1.262 = 0.86 μM/ml
280	.278	

Thus  $\frac{11.1}{28.6} = 39\%$  of AMP = Ad-Met

Yield of AMP ppt was  $2.1 \times \frac{28.6}{49} \times 650 = 797 \mu\text{M}$  or ca 80%

## Preparation of DCC

Copied from Khwara Notebook

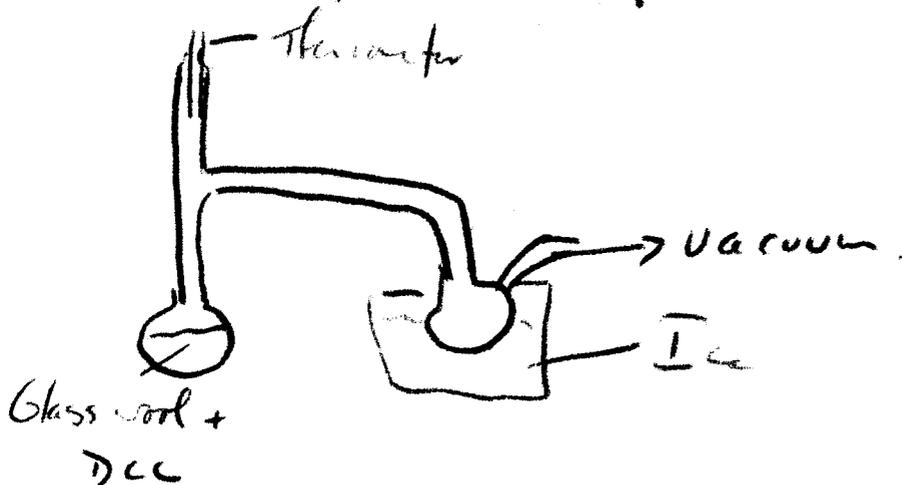
### Prep of dicyclohexylthiourea

600cc dicyclohexylamine to 400 cc ethyl alcohol. Add CS<sub>2</sub> (200 cc) carefully in very small portions and under constant shaking and cooling. After completion heat the almost solid contents for 1 1/2 days till H<sub>2</sub>S evolution is complete. The finely divided dithio carbonate salt is replaced by chunky plates of dicyclohexylthiourea. Stood for another day. Filter & wash with alcohol.



### Prep of DCC

50 gms of finely divided (powdered) dicyclohexylthiourea in ca 250-300 ml of CS<sub>2</sub> + 100g H<sub>2</sub>O powder placed mechanically for ca 6-7 hours then filter at wash with CS<sub>2</sub> in small amounts (also can use Pet ether) Clear filtrate evaporate to oil. Dissolve in ca 150 ml of pet ether & dry over H<sub>2</sub>SO<sub>4</sub> 24 hours. Filter & remove pet ether. Distill in vacuo (a few mm at ca 100°). Collect in short air condenser.



Yield when I did it was 25% but had been as high as 50-80%?

7/23/56

Stability of The Crude Adenyl - Methionine

Dissolved 50mg of The adenyl-methionine (crude 7/19) in 2ml H<sub>2</sub>O. pH was 3.2 @ glass electrode

0.1ml gave 0.350 O.D. @ H<sub>2</sub>SO<sub>4</sub> + FeCl<sub>3</sub>.

After 4 hours in ice bath 0.1ml gave 0.352 ∴ No degradation occurred under these conditions.

Placed remainder at rm temperature and removed samples at various times

	D <sub>540</sub> /0.1ml	
60'	.312	
135'	.251	
180'	~ .223	Beckman very unsteady

Seems to be degraded about 10% per hour

On 8/2/56 dissolved 50mg in 2ml cold H<sub>2</sub>O.

0.1ml gave D<sub>540</sub> .353

This material seems to be fairly stable in two weeks no breakdown.

## Attempted prep of Adenyl Methionine with di-p-tolyl carbodiimide

55mg Asp (.16M) + 30mg L-methionine (.20M) mixed with 0.25 ml H<sub>2</sub>O. Then added 0.95 ml pyridine followed by 0.03 ml 3M HCl. Stirred in an ice bath. Added 1 gm di-p-tolyl carbodiimide dissolved in 1 ml pyridine. As soon as added the rx mixture solidified. Added 1 ml pyridine and some of the material dissolved the mixture becoming liquid. Removed samples at 1 & 2 hrs. There was no formation of methionine hydroxamic acid.

D<sub>2</sub>O 0.002 & 0.005

One thing that appeared different was when an aliquot was added to the aqueous hydroxylamine there was a reddish brown ppt. which separated out. Could this be the acyl urea of methionine + di-p-tolyl urea.

7/24/56

Prep of Adenyl-Methionine

350mg ASP + 165mg L-methionine mixed with 1.1 ml H<sub>2</sub>O. Added 4.5 ml pyridine. Added 0.15 ml 8M HCl. Added 4 gm DCC dissolved in 4 ml pyridine. Stirred in ice bath. Removed 0.05 ml samples for assay.

<u>Time</u>	<u>DSV</u>	<u>μM/ml</u>	Estimated volume of rx mixture 13-14 ml.
60'	.825	48.5	
165'	1.207	71.0	$\times 13-14 = 923-995 \mu M$

At 180 minutes added 10 ml of acetone -20° and stirred for 1-2 minutes then placed at -5° for ca 5 minutes. Filtered ppt by suction at room temp. Took about 15 minutes to filter, then washed with 60 ml of 70% acetone alcohol (-20°C) in small portions. Then washed with 30 ml of ether (-20°) and sucked partially dry. ~~Disrupted~~ Suspended material in funnel in 5 ml ice water by washing then sucked dry. Repeated 2 or 3 more of cold H<sub>2</sub>O then washed filter with 1 ml H<sub>2</sub>O.

<u>Assay</u>	<u>DSV</u>	<u>μM/ml</u>
0.05 ml	.620	40

Assayed again on 8/2/56  
 $\frac{0.1 \text{ ml}}{.735} = \frac{\mu M/ml}{21.6}$   
 ~ 50% degraded in ca 9 days.

Diluted	0.03 ml → 25 ml
λ	0.0
250	1.204
253	1.434
257	1.550
260	1.415
265	1.325
280	.292

$\frac{250}{260} = 0.85$   
 $\frac{240}{260} = 0.21$

$\frac{1.329}{14.7} \times \frac{25}{.03} = \underline{\underline{75.3 \mu M/ml}}$

Correcting for pyridine content

$\frac{272}{\times 2} = 0.22$

$\frac{40}{75.3} = 53\%$

7/26/57

Trip of Adenyl-Meth put Ppt in 80% Alcohol 20% Ether

350 mg ASP + 165 mg L-meth mixed with 1.1 ml H<sub>2</sub>O. Then added 4.5 ml pyridine followed by 0.15 ml 3M HCl. Stirred in ice bath. Added 4 g DEC in 9 ml pyridine. Received 0.05 ml samples

Time	D <sub>540</sub>	µM/ml	
120'	1.130	66.5	69.3 x 13-14 = 900 - 970 ~ 90-97%
165'	1.175	69.3	

At 180 min added 110 ml of a 80:20 mixture of alcohol: ether (-15°C). Stirred in ice bath and after 5 min filtered in vacuum. Complete in ca 5 min. Washed with above solvent on filter. Then dissolved ppt in 11 ml cold H<sub>2</sub>O. All the ppt dissolved (the DEC should be soluble in the alcohol)

Assay vol 11-12 ml.  
 .03 → 25 ml 0.01 N HCl.

250	918	280/260 = .20	Concentration = $\frac{1.071}{14.3} \times \frac{25}{.03} =$
255	1.202		
257	1.212	250/260 = .25	62.8 µM/ml
260	1.176		
260	1.237		
			<u>Total</u>
			62.8 x 11-12 = 692 - 755 µM

<u>Hydroxamic Acid</u>	<u>D<sub>540</sub></u>	<u>µM/ml</u>	<u>Total</u>
.05 ml	.553	32.6	358 - 391

Purity  $\frac{32.6}{62.8} = 52\%$

On 8/2	0.10 ml	.545	16
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About 50% left after 8 days.  
kept frozen

7/27

## Effect of HCl on Synthesis of Acetyl Methionine

55g ASP + 30g L-methionine mixed in 0.25ml H<sub>2</sub>O. Added  
 0.85ml pyridine stir in ice then add 0.9g DCC dissolved  
 in 1ml pyridine volume about 2.5ml. Remove 0.05ml samples

<u>Time</u>	<u>D<sub>540</sub></u>	<u>μg/ml</u>
65'	.155	9.1
120'	.185	10.9
200'	.350	20.6

Total about 52-62 μM

Theory 160 μM

Thus in over three hours went about 30%. Seems as if  
 the acid does speed up the reaction or maybe the rate of  
 breakdown caught up to the rate of synthesis is increased